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Invention: TRANSGENIC PLANTS CARRYING NEOXANTHIN CLEAVAGE ENZYME GENE

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SPECIFICATION

TRANSGENIC PLANTS CARRYING NEOXANTHIN CLEAVAGE ENZYME GENE

FIELD OF THE INVENTION

The present invention relates to a DNA for improving or reducing stress tolerance in a plant, and a transgenic plant of the DNA.

BACKGROUND OF THE INVENTION

Plants must adapt themselves to various stresses, for example, drought, salt in the soil, and low temperature because they can not move freely. Among these stresses, drought is thought to effect plant growth the most severely. In order to survive in drought condition, some plants have acquired a physiologically and/or morphologically specific trait in the evolutional process while many other plants also confer a mechanism to response to the drought stress and defend themselves. These responses to a shortage of water and adaptation to drought environment in plants are caused by various physiological changes including the alternation of gene expression at drought (Shinozaki, K and Yamaguchi-Shinozaki, K., Plant Physiol., 115: 327-334, 1997; Shinozaki, K. and Yamaguchi-Shinozaki, K., "Molecular responses to drought stress." *In* Shinozaki and Yamaguchi-Shinozaki (eds), "Molecular responses to cold, drought, heat and salt stress in higher plants," R. G. LANDES company, Austin, Texas, USA, pp. 11-28, 1999). For example, in *Arabidopsis* (*Arabidopsis thaliana*), it is known that a drought signal is transmitted through an abscisic acid (ABA) dependent pathway and ABA independent pathway to control the gene expression involved in drought tolerance. These gene products are thought to have a function in controlling, for example, accumulation of osmoprotectants such as sucrose and proline, half life of proteins, stress signal transduction pathway, and transcription (Bray, E. A., Trends in Plant Science, 2: 48-54, 1997; Bohnert, H. J. et al., Plant Cell, 7: 1099-1111, 1995; Ingram, J. and Bartels, D., Annu. Rev. Plant Physiol. Plant Mol. Biol., 47: 377-403, 1996; Shinozaki, K. and Yamaguchi-Shinozaki, K., Plant Physiol., 115:

327-334, 1997; Shinozaki, K. and Yamaguchi-Shinozaki, K., "Molecular responses to drought stress." *In* Shinozaki and Yamaguchi-Shinozaki (eds), "Molecular responses to cold, drought, heat and salt stress in higher plants," R. G. LANDES company, Austin, Texas, USA, pp. 11-28, 1999).

C40 pathway has been proposed as a biosynthetic pathway of ABA in higher plants. The C40 pathway, also called a carotenoid pathway, is a synthetic pathway through epoxydation of zeaxanthin, synthesizing violaxanthin, neoxanthin, xanthoxin, ABA aldehyde, and then ABA (Zeevaart, J. A. D. and Creelman R. A., Ann. Rev. Plant Physiol. Plant Mol. Biol., 39: 439-473, 1988). This biosynthetic pathway has been proposed from physiological studies and analyses of ABA biosynthetic variants. For example, variant *aba2* isolated from tobacco (*Nicotiana tabacum*) has a mutation in a gene (*aba2*) of zeaxanthin epoxidase enzyme which catalyzes the epoxidation of zeaxanthin (Marin E. et al., EMBO J., 15: 2331-2342, 1996). Variant *vp14* isolated from maize has a mutation in a gene (*VP14*) of neoxanthin cleavage enzyme which catalyzes the conversion from a neoxanthin to xanthoxin (Tan, B. C. et al., Proc. Natl. Acad. Sci. USA, 94: 12235-12240, 1997). From *Arabidopsis* plants, variant *aba3* having a mutation in an enzyme which catalyzes a reaction from xanthoxin to ABA aldehyde, and variant *aba4* involved in the reaction for oxidizing ABA aldehyde to produce ABA have been isolated (Schwartz, S. H. et al., Plant Physiol., 114: 161-166, 1997; Leon-Kloosterziel, K. M. et al., Plant J., 10: 655-661, 1996).

A maize having a mutation in a neoxanthin cleavage enzyme gene (*VP14*) is known to show a trait of easily loosing water and easily wilting. It has not been known yet, however, whether stress tolerance in plants can be improved or not using the neoxanthin cleavage enzyme gene.

SUMMARY OF THE INVENTION

An objective of the present invention is to provide a DNA encoding a neoxanthin cleavage enzyme used for improving stress tolerance in a plant, a method for increasing stress tolerance

in a plant by introducing the DNA into the plant, and a transgenic plant into which a neoxanthin cleavage enzyme gene is introduced. Another objective of the present invention is to provide a DNA used for reducing stress tolerance in a plant, a method for decreasing stress tolerance in a plant by introducing the DNA into the plant, and a transgenic plant into which the DNA is introduced. The improvement of stress tolerance in plants is useful, for example, in plant breeding.

The present inventors have isolated a cDNA clone (CPRD65) corresponding to a gene involved in a response against drought treatment, by the differential screening of a cDNA library prepared from a cowpea plant (*Vigna unguiculata*) which showed extensive drought tolerance after dehydration treatment for 10 hours. The CPRD65 cDNA was expected to encode a neoxanthin cleavage enzyme proposed to be involved in biosynthesis of abscisic acid (ABA). Drought stress given to an 8-day-old cowpea plant strongly induced the accumulation of ABA and the expression of the CPRD65, indicating the potential of the profound involvement of CPRD65 gene, especially in the response to drought stress. Determination of an enzyme activity using GST-CPRD65 fusion protein confirmed that the CPRD65 comprises an activity of cleaving 9-*cis*-neoxanthin to produce xanthoxin. These results indicate that the CPRD65 gene encodes a neoxanthin cleavage enzyme and its product plays a key role in endogenous ABA biosynthesis under drought stress.

Moreover, the present inventors have isolated a novel gene (AtNCED3) by screening a neoxanthin cleavage enzyme gene from an *Arabidopsis* plant-derived cDNA library using a cDNA of the CPRD65 gene isolated from cowpea plants as a probe. In addition, four types of sequences (AtNCED1, 2, 4, and 5) derived from an *Arabidopsis* plant comprising high homology with these genes were identified. Expression of these genes in *Escherichia coli* (*E. coli*) and assay of a neoxanthin cleavage activity revealed that AtNCED1, 3, and 5 comprise a neoxanthin cleavage enzyme activity same as the CPRD65.

The present inventors first produced a transgenic plant of

Arabidopsis using AtNCED3, a neoxanthin cleavage enzyme gene. The AtNCED3 gene was ligated downstream of 35S promoter in a vector for introducing a gene into plant cells (pBE2113N) in the directions of sense (an overexpression type) or antisense (an expression inhibition type) and introduced the vector into *Arabidopsis* by the vacuum infiltration method. Evaluation of drought tolerance of the prepared transgenic plants revealed that stress tolerance in the overexpressed plants was significantly increased compared with that in their parent lines. In contrast, in the expression-inhibited lines into which the antisense was introduced, stress tolerance was reduced (Figs. 15 and 16). In such a manner, the present inventors found that actually the transgenic plant into which the neoxanthin cleavage enzyme gene is introduced significantly increased stress tolerance and stress tolerance can be significantly reduced by decreasing the expression of the gene to complete the present invention.

Specifically, this invention relates to a DNA encoding a neoxanthin cleavage enzyme used for improving stress tolerance in a plant, a method for increasing stress tolerance in a plant by introducing the DNA into the plant, and a transgenic plant into which a neoxanthin cleavage enzyme gene is introduced, as well as a DNA used for reducing stress tolerance in a plant, a method for decreasing stress tolerance in a plant by introducing the DNA into the plant, and a transgenic plant into which the DNA is introduced, and more specifically, the present invention provides:

(1) an isolated DNA encoding a protein having a neoxanthin cleavage activity for improving stress tolerance in a plant,

(2) an isolated DNA for reducing stress tolerance in a plant, wherein the DNA is selected from the group consisting of:

(a) a DNA encoding an antisense RNA complementary to a transcript of a gene encoding a protein having a neoxanthin cleavage activity;

(b) a DNA encoding an RNA comprising a ribozyme activity which cleaves a transcript of a gene encoding a protein having a neoxanthin cleavage activity; and

(c) a DNA encoding an RNA which inhibits the expression of a gene

encoding a protein having a neoxanthin cleavage in a plant cell by the cosuppression effect,

(3) the DNA of (1) or (2), wherein the protein having a neoxanthin cleavage activity is selected from the group consisting of:

(a) a protein comprising an amino acid sequence of SEQ ID NOs: 2 (AtNCED1), 6 (AtNCED3), 10 (AtNCED5), 12 (CPRD65), 14 (VP14), or 16 (LeNCED1),

(b) a protein comprising an amino acid sequence in which one or more amino acids in SEQ ID NOs: 2 (AtNCED1), 6 (AtNCED3), 10 (AtNCED5), 12(CPRD65), 14 (VP14), or 16 (LeNCED1) are replaced, deleted, added, and/or inserted, and

(c) a protein encoded by a DNA which hybridizes with a DNA comprising a nucleotide sequence of SEQ ID NOs: 1 (AtNCED1), 5 (AtNCED3), 9 (AtNCED5), 11 (CPRD65), 13 (VP14), or 15 (LeNCED1) under the stringent condition,

(4) the DNA of any one of (1) to (3), wherein the protein having a neoxanthin cleavage activity is derived from *Arabidopsis* plants,

(5) a transformant plant cell carrying the DNA of any one of (1) to (4),

(6) a transgenic plant comprising the transformant plant cell of (5),

(7) a transgenic plant which is offspring or a clone of the transgenic plant of (6),

(8) the transgenic plant of (6) or (7), wherein the expression of a gene encoding a protein having a neoxanthin cleavage activity is increased or decreased compared with its wild type,

(9) the transgenic plant of any one of (6) to (8), wherein the amount of abscisic acid is increased or decreased compared with its wild type,

(10) the transgenic plant of any one of (6) to (9), wherein stress tolerance is increased or decreased compared with its wild type,

(11) a propagation material for the transgenic plant of any one of (6) to (10),

(12) a vector comprising the DNA of any one of (1) to (4),

(13) a method for producing the transgenic plant of any one of

(6) to (10), comprising the steps of introducing the DNA of any one of (1) to (4) into a plant cell and regenerating a plant from the plant cell,

(14) a method for increasing or decreasing stress tolerance in a plant, comprising expressing the DNA of any one of (1) to (4) in a plant cell,

In the present invention, "stress tolerance" means tolerance against environmental stresses, for example, drought stress tolerance, salt stress tolerance, low temperature stress tolerance, air pollution tolerance, tolerance to low oxygen condition, pathogen tolerance, drug tolerance such as those to agrochemicals, etc. Exogenous treatment with ABA has known to improve tolerance against these stresses in many plants (refer to Takahashi, N. and Masuda, Y. (eds), "Plant Hormone Handbook (The Last)," Baifukan, Japan, pp. 78-160; and references cited therein).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows Northern blot analysis of the expression of the CPRD65 genes upon dehydration or rehydration. Total RNA was prepared from 8-day-old cowpea plants that had been dehydrated for 0, 1, 2, 4, 6, 8, 10, and 12 hours or rehydrated for 0, 1, 2, 5, 10, and 24 hours after dehydration for 10 hours. Each lane was loaded with 10 μg of total RNA. The RNA was fractionated on a 1% agarose gel, blotted onto a nylon membrane, and probed with [^{32}P]-labeled cDNA inserts of the CPRD65 clones.

Figure 2 shows comparison of the deduced amino acid sequences of the CPRD65, VP14 (neoxanthin cleavage enzyme from *Zea mays*, Schwartz, S. H. et al., Science, 276: 1872-1874, 1997), and LeNCED1 protein (neoxanthin cleavage enzyme from *Lycopersicon esculentum*, Burbidge, A. et al., J. Exp. Bot., 47: 2111-2112, 1997; Burbidge, A. et al., Plant J., 17: 427-431, 1999). Dashes indicate gaps that were introduced to optimize the alignment. Enclosed boxes indicate identical amino acids. Shadowed regions indicate similar amino acids.

Figure 3 shows Southern blot analysis of genomic DNA from

cowpea 2246 cultivar. Genomic DNA (10 μg per lane) was digested with EcoRI (E), HindIII (H), and XbaI (X), fractionated on a 1% agarose gel, and transferred to a nylon membrane. The filter was allowed to hybridize with a [^{32}P]-labeled fragment of the CPRD65 cDNA. "A" and "B" represent different stringency in hybridization conditions (refer to Examples). The size marker of DNA fragments is indicated in kbp.

Figure 4 (A) shows Northern blot analysis of the induction of the CPRD65 gene by high salinity (NaCl), high temperature (heat), low temperature (cold), and the application of abscisic acid (ABA). Total RNA was isolated from the cowpea plants at the indicated hours after the treatment. Each lane was loaded with 10 μg of total RNA. The number above each lane indicates the duration (hours) of the treatment.

Figure 4 (B) shows Northern blot analysis of the CPRD65 gene without or with 10 hour-dehydration treatment. Each lane was loaded with 10 μg of total RNA isolated from leaves (L), stems (S), and roots (R) of cowpea 2246 cultivar. The RNA was fractionated on a 1% agarose gel, blotted onto a nylon membrane, and probed with [^{32}P]-labeled cDNA inserts of the CPRD65.

Figure 5 shows HPLC profiles of carotenoid metabolites of GST (A) or the GST-CPRD65 recombinant protein (B). The reaction mixture contained *cis*-neoxanthin as a substrate. cN; *cis*-neoxanthin, C25; C25-product.

Figure 6 shows plastid targeting of the CPRD65N-sGFP chimeric protein in protoplasts. Constructs carrying the 35S-sGFP (A, C, E) or the 35S-CPRD65N-sGFP chimeric constructs (B, D, F) were transfected into *A. thaliana* protoplasts using polyethylene glycol (PEG). Transfected protoplasts were observed by optical microscopy (A, B) or fluorescent microscopy with an interference filter type green (E, F) or red (C, D). E and F indicate GFP localization, and C and D chloroplast.

Figure 7 shows the relationship between the accumulation of ABA and the expression of the gene for CPRD65 during dehydration. The radioactivity retained on the nylon filter in Fig. 1 was quantified and plotted as shown. The procedure for

quantification of ABA is described in Examples. Error bars show standard errors. The experiment was repeated three times.

Figure 8 shows the accumulation of endogenous ABA in leaves (L), stems (S), and roots (R) of cowpea plants during dehydration treatment after separation of organs. The procedure for quantification of ABA is the same as described in Example 7 (Figure 7).

Figure 9 shows comparison of the deduced amino acid sequences of AtNCED3 and CPRD65. Dashes indicate gaps that were introduced to optimize the alignment. Enclosed boxes indicate identical amino acids. Shadowed regions indicate similar amino acids.

Figure 10 shows alignments of amino acid sequences of AtNCED1, 2, 3, 4, and 5. Dashes indicate gaps that were introduced to optimize the alignment. Enclosed boxes indicate identical amino acids. Shadowed regions indicate similar amino acids.

Figure 11 shows the result of the phylogenic analysis to examine relationship between the amino acid sequences of AtNCED1, 2, 3, 4, 5, and CPRD65, and their related sequences on the databases. LeNCED1 (Ac. No. Z97215) and VP14 (Ac. No. U95953) show the proteins derived from tomatoes and maize, respectively.

Figure 12 shows the expression of the AtNCED genes against each stress.

Figure 13 shows the expression of the AtNCED3 gene in AtNCED3-transformants. The upper and lower panels show the expression of the AtNCED3 gene in plants before drought and after drought stress treatment, respectively. Two strains were used for both of plants expressing the sense AtNCED3 gene (overexpression) (A and B) and plants expressing the antisense (expression inhibition) (C and D).

Figure 14 shows endogenous ABA amounts in AtNCED3-transformants. Endogenous ABA amounts increased in plants expressing the sense AtNCED3 gene (overexpression) (A and B) but decreased in those expressing the antisense (expression inhibition) (C and D), compared with wild type plants.

Figure 15 shows the result of testing drought tolerance in

neoxanthin cleavage enzyme transgenic plants, indicating the plants and relative water content in the leaves 14 days after the termination of the irrigation.

Figure 16 shows the result of testing drought tolerance in neoxanthin cleavage enzyme transgenic plants, indicating the plants 17 days after the termination of the irrigation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an isolated DNA encoding a protein having a neoxanthin cleavage activity used for improving stress tolerance. A neoxanthin cleavage enzyme has been known as an enzyme involved in the ABA biosynthesis, however, has not been confirmed whether introduction of the DNA encoding this enzyme into a plant actually leads to ABA accumulation and improvement of tolerance against stresses without a grave effect to plant's growth.

Exogenous treatment with ABA causes, for example, growth inhibition in many plants. In a seed, it is known that ABA also causes growth inhibition (germination inhibition) (Takahashi, N. and Masuda, Y. (eds), "Plant Hormone Handbook (The Last)," Baifukan, Japan, pp. 78-160; and references cited therein). Increase in ABA level brings about various damages to plants. There has been no report whether excessive production of ABA by an exogenous gene leads to acquirement of stress tolerance or not. The conventional experimental procedures for exogenous treatment with ABA require the treatment at high concentration, which strongly inhibits the growth and has prevented accurate evaluation of tolerance. Furthermore, experiments of exogenous treatments have not identified that an appropriate level of ABA ensures normal growth and acquirement of tolerance. By obtaining ABA biosynthesis gene and creating a transgenic plant using this gene, the present inventors have first confirmed that stress tolerance in a plant can be improved.

An "isolated DNA" is a DNA the structure of which is not identical to that of any naturally occurring DNA or to that of

any fragment of a naturally occurring genomic DNA spanning more than three separate genes. The term therefore includes, for example, (a) a DNA which has the sequence of part of a naturally occurring genomic DNA molecule in the genome of the organism in which it naturally occurs; (b) a DNA incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein. Specifically excluded from this definition are DNA molecules present in mixtures of different (i) DNA molecules, (ii) transfected cells, or (iii) cell clones; e.g., as these occur in a DNA library such as a cDNA or genomic DNA library.

As an isolated DNA used for increasing stress tolerance, any genes can be used as long as it encodes a protein having a neoxanthin cleavage activity. For example, VP14 of maize (*Zea mays*) (Schwartz, S. H. et al., Science, 276: 1872-1874, 1997; Tan, B. V. et al., Proc. Natl. Acad. Sci. USA, 94: 12235-12240, 1997) (cDNA: SEQ ID NO: 13, protein: SEQ ID NO: 14), LeNCED1 of tomato *(Lycopersicon esculentum)* (Burbidge, A. et al., J. Exp. Bot., 47: 2111-2112, 1997; Burbidge, A. et al., Plant J., 17: 427-431, 1999) (cDNA: SEQ ID NO: 15, protein: SEQ ID NO: 16), and such have been isolated as neoxanthin cleavage enzyme genes. These genes are useful for improving stress tolerance and can be used for the present invention. In addition, DNAs encoding AtNCED1 (SEQ ID NO: 2), AtNCED3 (SEQ ID NO: 6), AtNCED5 (SEQ ID NO: 10), and CPRD65 (SEQ ID NO: 12) (SEQ ID NOs: 1, 5, 9, and 11, respectively) can be conveniently used. Moreover, a DNA encoding a neoxanthin cleavage enzyme of SEQ ID NO: 18 (cDNA: SEQ ID NO: 17, protein: SEQ ID NO: 18) (Neill, S. J. et al., J. Exp. Bot., 49: 1893-1894, 1998, Ac. No. AJ005813) can also be used in this invention. A DNA encoding a protein having a neoxanthin cleavage activity can

also be used as a reagent for increasing stress tolerance (stress tolerance increasing agent). The present invention also provides uses of a DNA encoding a protein having a neoxanthin cleavage activity for increasing stress tolerance.

A DNA encoding a protein having a neoxanthin cleavage activity in the present invention includes a genomic DNA, a cDNA, and a chemosynthetic DNA. A genomic DNA and a cDNA can be prepared by common methods for one skilled in the art. A genomic DNA can be prepared, for example, by extracting a genomic DNA from a plant according to conventional methods, in which a genomic library is prepared(in which, as a vector, for example, a plasmid, a phage, a cosmid, and a BAC can be used), and colony hybridization or plaque hybridization is conducted using a probe based on the DNA of the present invention (for example, SEQ ID NOs: 1, 5, 9, 11, 13, 15, etc.). Alternatively, a genomic DNA can also be prepared by conducting PCR with primers specific to the DNA of the present invention (for example, SEQ ID NOs: 1, 5, 9, 11, 13, 15, etc.). A cDNA can be prepared by synthesizing a cDNA based on a mRNA extracted from a plant, inserting the cDNA into a vector, such as λ phage to prepare and develop a cDNA library, and conducting colony hybridization or plaque hybridization in the same manner as above, or performing PCR. A DNA of the present invention includes not only DNA sequences of SEQ ID NOs: 1, 5, 9, 11, 13, and 15, but a DNA comprising nucleotide sequences based on an optional degeneracy of codons encoding amino acids of each protein.

A DNA of the present invention also includes, for example, a DNA encoding a protein that comprises an amino acid sequence in which one or more amino acids are replaced, deleted, added, and/or inserted in SEQ ID NOs: 2, 6, 10, 12, 14, or 16, and has a neoxanthin cleavage activity. Thus, the DNA of the invention includes a mutant, a derivative, an allele, a variant, and a homolog of SEQ ID NOs: 1, 5, 9, 11, 13, or 15, a gene derived from a natural plant.

A modified protein encoded by the DNA of the invention

comprises an amino acid sequence at least 70% (e.g., 80%, 90%, 95%, or 99%) identical to SEQ ID NOs: 2, 6, 10, 12, 14, or 16. As used herein, "percent identity" of two amino acid sequences or of two nucleic acids is determined using the algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87: 2264-2268, 1990) modified as in Karlin and Altschul (Proc. Natl. Acad. Sci. USA 90:5873-5877, 1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al. (J. Mol. Biol. 215:403-410, 1990). BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12. BLAST protein searches are performed with the XBLAST program, score = 50, wordlength = 3. Where gaps exist between two sequences, Gapped BLAST is utilized as described in Altsuchl et al. (Nucleic Acids Res. 25: 3389-3402, 1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g, XBLAST and NBLAST) are used. See http://www.ncbi.nlm.nih.gov.

The modified protein in which one or more amino acids are replaced in SEQ ID NOs: 2, 6, 10, 12, 14, or 16 is preferably obtained by at least one conservative amino acid substitution. A "conservative amino acid substitution" is a replacement of one amino acid residue belonging to one of the following groups having a chemically similar side chain with another amino acid in the same group. Groups of amino acid residues having similar side chains have been defined in the art. These groups include amino acids with basic side chains (e.g., lysine, arginie, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, aspargine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

An example of a method for preparing such a DNA encoding

a protein having a modifiend amino acid sequence, well-known to one skilled in the art, is *in vitro* mutagenesis utilizing PCR (Izawa, T., "*in vitro* mutagenesis by PCR" in Shimamoto, K. and Sasaki, T. (supervisors), Cell Technology, Supplement, Plant Cell technology Series VII, Protocols for PCR Experiments in Plants, New Edition, pp. 151-158, Shujunsha, Japan). Modification of amino acids in a protein, is ordinarily within 200 amino acids, preferably within 100 amino acids, more preferably within 50 amino acids, and further more preferably within 10 amino acids in the case of artificial modification. Modification of an amino acid sequence of a protein due to modification of the encoding nucleotide sequence can occur in nature. A DNA encoding a protein having an amino acids sequence in which one or more amino acids are replaced, deleted, added, and/or inserted in an amino acid sequence encoding a wild-type neoxanthin cleavage enzyme is even included in the DNA of the present invention as long as it encodes a protein having a neoxanthin cleavage activity. The DNA of the present invention also includes a degenerate variant in which a mutation in a nucleotide sequence does not result in a mutation of amino acids in a protein.

Whether a given DNA encodes a neoxanthin cleavage enzyme or not can be determined by expressing the DNA in *E. coli* to prepare a recombinant protein and detecting the cleavage using a *cis*-neoxanthin as a substrate, according to Example 5 below.

Based on a DNA encoding a known neoxanthin cleavage enzyme, a novel neoxanthin cleavage enzyme gene can be isolated. Examples of methods well-known by one skilled in the art for this purpose are methods using hybridization technique (Maniatis, T et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press) and polymerase chain reaction (PCR) techniques (Nakayama, H., Cell Technology, Supplement, Biological Experiment Illustrated, Vol. 3, New Edition, Shujunsha, 1998). Specifically, one skilled in the art can routinely isolate a DNA encoding a neoxanthin cleavage enzyme gene from any plant by using a nucleotide sequence of a known neoxanthin cleavage enzyme gene (for example, SEQ ID NOs: 1, 5,

9, 11, 13, 15, etc.) or its partial sequence as a probe, as well as an oligonucleotide specifically hybridized with these sequences as a primer. A DNA encoding a neoxanthin cleavage enzyme, capable of being isolated by such hybridization technique or PCR technique is also included in a DNA used for improving stress tolerance in the present invention.

Hybridization can be performed under stringent condition by following, for example, the method described in reference (Sambrook, J., et al., "Molecular Cloning: A Laboratory Manual" 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) using a [^{32}P]-labeled DNA prepared by using a random prime method as a probe. A DNA is blotted to a nylon membrane and hybridized with a [^{32}P]-labeled fragment, for example, in a hybrudization solution containing 30%, preferably 50% formamide, 6X SSC, 5X Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA, at 37°C, preferably at 42°C. Under a stringent condition, washing is, for example, in 1X SSC, 1% SDS (room temperature), for 15 min twice, preferably (more stringent) in 0.5X SSC, 0.5% SDS (37°C), for 15 min twice, and more preferably (further stringent) 0.1X SSC, 0.1% SDS (60°C) for 15 min twice, and subjected to autoradiography. By "hybridizes under stringent conditions" is meant specific and non-covalent equilibrium binding by base-pairing to an immobilized reference nucleic acid under the above conditions.

In order to prepare a transgenic plant with improved stress tolerance using these DNAs, the DNA is inserted into an appropriate vector, and introduced the vector into a plant cell, and a transgenic plant is regenerated from the transformant plant cell.

As a vector used for transformation of a plant cell, any vector capable of expressing an inserted gene in the cell can be used. For example, a vector comprising a promoter for constantly expressing a gene in a plant cell (for example, 35S promoter of cauliflower mosaic virus) and a vector with a promoter inducibly activated by an exogenous stimulus can be used. Alternatively, by using a promoter specific to a plant tissue to induce the expression of an objective gene, stress tolerance can be provided

specifically to a tissue highly sensitive to the stress. For example, an objective gene can be expressed specifically to a tissue by using a promoter of a gene specifically expressing in seeds, such as that of β-phaseolin gene of kidney beans (Bustos et al., EMBO J., 10: 1469-1479, 1991) and that of glycinin gene of soybean (Lelievre et al., Plant Physiol., 98: 387-391, 1992), a promoter of a gene expressing specifically in leaves, such as that of RbcS gene of pea (Lam and Chua, Science, 248: 471-474, 1990) and that of Cab1 gene of wheat (Gotorn et al., Plant J., 3: 509-518, 1993), and a promoter of a gene expressing specifically to roots, such as that of TobRB7 gene of tobacco (Yamamoto et al., Plant Cell, 3: 371-382, 1991), etc. Alternatively, a vector comprising a promoter inducibly activated by an exogenous stimulus can be used. An example of a promoter responding to environmental stresses, such as drought, salt, or low temperature, is a promoter of rd29A gene (Yamaguchi-Shinozaki, K. and Shinozaki, K., Plant Cell, 6: 251-264, 1994). A promoter to be activated by environmental stresses such as drought and high salt concentration is also preferably used in the present invention. Examples of such promoters are those of *Arabidopsis* AtNCED3 gene, cowpea CPRD65 gene, and so on. Moreover, by using an expression system inducible by a drug, an objective gene can be expressed at an optional timing and in an optional tissue. An example of an expression system induced by steroid hormone (glucocorticoid) is an induction system using GVG gene (GAL4, VP16, Glucocorticoid receptor) (Aoyama T. and Chua, N. H., Plant J, 11: 605-12, 1997).

A vector can be inserted into any plant cells, for example, *Arabidopsis* (*Arabidopsis thaliana*), rice (*Oriza sativa*), tobacco (*Nicotiana tabacum*), tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*), maize (*Zea mays*), bird's foot trefoil (*Lotus japonicus*), and so on. Other crops or trees are also useful. A plant can be a conifer, a broad-leaved tree, a dicot, a monocot, etc. "Plant cell(s)" used herein include various forms of plant cells, for example, a suspended cultured cell, a protoplast, a leaf slice, a callus, etc.

For introduction of a vector into a plant cell, various

methods known to one skilled in the art, for example, polyethylene glycol method, electroporation method, agrobacterium method, vacuum infiltration method, particle gun method, and such can be applied. A plant can be regenerated from a transformant plant cell by methods well-known to one skilled in the art, depending on a type of a plant cell. For example, a transformant of rice, *Arabidopsis*, or such can be prepared according to the method described in "Simamoto, K., Okada K. (supervisors), Cell Technology, Supplement, Plant cell Technology Series 4, Experimental Protocol for Model Plants, Shujunsha, Japan."

Once a transformant plant into which genome the DNA of the present invention is introduced is obtained, offspring can be obtained by sexual or asexual propagation from the plant. Alternatively, a propagation material (for example, a seed, a fruit, a scion, a tuber, a tuberous root, a stock, a callus, a protoplast, etc.) is obtained from the plant, its offspring, or clones, and the plant can be mass-produced from them. The present invention includes a plant cell into which the DNA of the present invention is introduced, a plant containing the cell, offspring or a clone of the plant, as well as propagation materials for the plant, its offspring, and clone.

A transformant plant produced in such a manner has an increased ABA content, compared with its wild type plant. Alternatively, a transformant plant has improved stress tolerance, compared with its wild type plant. Stress tolerance can be compared by a known method. For example, as described in Examples below, a plant is grown under the stress condition, such as drought, high salt, low temperature, or heat condition, and the growth of individuals is compared. For example, comparison can be made by measuring an appearance, a size of a plant or of a tissue such as a leaf, a stem, and a root, a weight (wet weight or dry weight), color, a relative growth rate, a photosynthetic activity, etc. as an index. Stress tolerance may increase in at least one tissue of a plant. A level of ABA in a plant can be determined by, for example, immunoassay, thin layer chromatography (TLC), gas chromatography (GC), and HPLC (refer to Takahashi, N. and Masuda,

Y. (eds), "Plant Hormone Handbook (The Last)," Baifukan, Japan, pp. 1-21; and the references cited therein). For example, as described in Example 7, reliable quantification is possible by finally quantifying crude purified fraction by HPLC with GC/MS, using labeled ABA as an internal standard.

By the present invention, a useful crop can be grown in an area exposed to environmental stress, for example, a drought zone, a cold zone, or high concentration of salt. In addition, the present invention can be applied to plants other than crops for tree-planting environment.

The present invention also relates to a DNA which can decrease the expression of a gene encoding a protein having a neoxanthin cleavage activity to be used for lowering stress tolerance. The DNA can also be used as a reagent for decreasing stress tolerance (stress tolerance decreasing agent). The present invention also provides uses of a DNA which can decrease the expression of a gene encoding a protein having a neoxanthin cleavage activity for decreasing stress tolerance.

A plant with reduced stress tolerance is useful for removing weeds and such from the environment, by applying to weeds and such. For example, a plant capable of inducing the decrease in stress tolerance can be prepared to apply for land improvement and such. To a plant with high regeneration ability, such as weed, a construct which inhibits the expression of a neoxanthin cleavage enzyme gene (for example, in the antisense direction) is introduced to downstream of a promoter inducible with a chemical (for example, glucocorticoid and so on). This transformant plant can normally grow without the application of the chemical. An arid land can be improved by growing the transformant weed for several years, spraying glucocorticoid to remove the weed at once by specifically lowering stress tolerance in the weed, and planting a crop plant. As a crop plant, a transformant crop which overexpresses a neoxanthin cleavage enzyme (a plant into which the DNA is introduced in sense direction) and such can be planted.

The present inventors first successfully created a transformant plant in which expression of a neoxanthin cleavage

enzyme is artificially inhibited by using a gene construct which expresses an antisense RNA of a neoxanthin cleavage enzyme gene. This plant easily wilts in the non-irrigated condition compared with its wild type to show the reduced stress tolerance (Figs. 15 and 16). In such a manner, the present inventors established a method for artificially inhibiting expression of a gene encoding a protein having a neoxanthin cleavage activity and successfully reduced stress tolerance in a plant thereby.

In the present invention, in order to reduce stress tolerance in a plant, the expression of a gene encoding a protein having a neoxanthin cleavage activity can be decreased. The term "expression" of gene used herein includes the transcription of the gene and the translation of the transcript. The inhibition of the expression includes the complete termination of the expression. To inhibit the transcription and the translation of the gene encoding a protein having a neoxanthin cleavage activity, the expression of the gene may be inhibited by targeting the DNA encoding the gene, its transcriptional control region, or the transcript of the gene.

Any plants can be used for the present invention, and various plants can be used. For example, *Arabidopsis* and such can be used. Examples of a gene encoding a protein having a neoxanthin cleavage activity, which can be a target for inhibiting the expression, are VP14 for maize (*Zea mays*) (Schwartz, S. H. et al., Science, 276: 1872-1874, 1997; Tan, B. V. et al., Proc. Natl. Acad. Sci. USA, 94: 12235-12240, 1997) (cDNA: SEQ ID NO: 13, protein: SEQ ID NO: 14), LeNCED1 for tomato (*Lycopersicon esculentum*) (Burbidge, A. et al., J. Exp. Bot., 47: 2111-2112, 1997; Burbidge, A. et al., Plant J., 17: 427-431, 1999) (cDNA: SEQ ID NO: 15, protein: SEQ ID NO: 16), AtNCED1 (cDNA: SEQ ID NO: 1, protein: SEQ ID NO: 2), AtNCED3 (cDNA: SEQ ID NO: 5, protein: SEQ ID NO: 6), and/or AtNCED5 (cDNA: SEQ ID NO: 9, protein: SEQ ID NO: 10) for *Arabidopsis*, CPRD65 (cDNA: SEQ ID NO: 11, protein: SEQ ID NO: 12) for cowpea, etc. Homologous genes derived from other plants can also be a target. Homologous genes of other plants can be identified and/or isolated by, for example, the hybridization

method described above and such. For reducing stress tolerance in a given plant in the present invention, using a gene or gene sequence information of another plant species (for example, the gene above), the expression of a target gene in the desired plant can be inhibited by known methods, such as gene silencing and antisense methods. Therefore, a target gene in an objective plant is not necessarily isolated nor identified.

The expression of a gene encoding a protein having a neoxanthin cleavage activity of the present invention can be inhibited by inserting a DNA for inhibiting the expression of the gene into an appropriate vector, introducing the vector into a plant cell, and regenerating a transgenic plant from the resultant transformant cell. Any promoters can be used, for example, the promoters as described above case for improving stress tolerance. For example, use of an expression inducible type promoter can reduce stress tolerance only under a specific condition.

As a method for inhibiting the expression of a specific endogenous gene in a plant, a method using the antisense technique is used the most often by one skilled in the art.

The antisense method is an artificial gene expression inhibition method in which a double strand of a target mRNA with a DNA molecule (an antisense nucleic acid) complementary to the RNA transcribed from a given gene forms for inhibiting the expression. The gene expression inhibition method by an antisense was developed from 1960 to 1970, and in 1978, Zamecnik et al. successfully inhibited the replication and reverse transcriptase activity of chicken Rous sarcoma virus using an antisense oligomer (Zamecnik, P. C. and Stephenson, M. L., Proc. Natl. Acad. Sci. USA, 75: 280-284, 1978).

Among methods for introducing an antisense DNA, an antisense oligomer is directly administered into a cell, or transformation is conducted by ligating an antisense DNA of a target gene with an expression vector. Examples given below demonstrate the latter method. Specifically, a cDNA of a gene encoding a protein having a neoxanthin cleavage activity is ligated downstream of 35S promoter of cauliflower mosaic virus

in the antisense direction to introduce the vector into a plant cell. The antisense effect in a plant cell was first demonstrated by Ecker et al. by showing the antisense effect of the antisense RNA introduced by an electroporation using a transient gene expression method in a plant (Ecker, J. R. and Davis, R. W., Proc. Natl. Acad. Sci. USA, 83: 5372, 1986). After that, inhibition of a target gene expression by an antisense RNA expression have been reported in tobacco and petunia plants (van der Krol, A. R. et al., Nature, 333: 866, 1988). At present, the antisense method is well established as a mean for inhibiting gene expression in a plant. Modes of inhibition of a target gene expression by an antisense nucleic acid include inhibition of transcription initiation by the formation of a triple strand, inhibition of transcription by the formation of a hybrid with a site at which an open loop structure is locally created by an RNA polymerase, inhibition of splicing by the formation of hybrid with an RNA in which a synthesis is occurring, inhibition of splicing by the formation of a hybrid with a spliceosome formation site, inhibition of transfer from a nucleus to a cytoplasm by the formation of a hybrid with an mRNA, inhibition of splicing by the formation of a hybrid with a capping site or a Poly (A) addition site, inhibition of translation initiation by the formation of a hybrid with a translation initiation factor binding site, inhibition of translation by the formation of a hybrid with a ribosome binding site flanking an initiation codon, inhibition of extension of a peptide strand by the formation of a hybrid with a coding region of mRNA or a polysome binding site, inhibition of gene expression by the formation of a hybrid with an interaction site between a nucleic acid and a protein, and so on. These inhibit processes of transcription, splicing, or translation to inhibit the expression of a target gene (Hirajima and Inoue, "New Biochemistry Experiment Lecture 2, Nucleic Acid IV, Replication and expression of a gene," Japanese Association of Biochemistry (eds), Tokyo-Kagakudojin, pp. 319-347, 1993).

A sequence of an antisense DNA is preferably a sequence complementary to a transcript of an endogenous gene encoding a

protein having a neoxanthin cleavage activity or its part in a plant to be transformed, however, is not necessarily completely complementary as long as it effectively inhibits the gene expression. An transcribed RNA comprises preferably 90% or higher complementarity, and the most preferably 95% or higher complementarity to the transcript of a target gene. For effectively inhibiting the expression of a target gene using an antisense sequence, a length of an antisense DNA is at least 15 or more nucleotides, preferably 100 or more nucleotides, and more preferably 500 or more nucleotides. Generally, an antisense DNA to be used is shorter than 5 kb and preferably shorter than 2.5 kb.

The expression of an endogenous gene can also be inhibited by using a DNA encoding a ribozyme. Recently, the inhibition of a gene expression using a DNA encoding a ribozyme has been studied. A ribozyme is an RNA comprising an activity of catalyzing a reaction *in vivo*. Ribozymes have various activities. The studies of a ribozyme as an enzyme cleaving an RNA enable designing a ribozyme for the purpose of cleaving an RNA at a specific site. Ribozymes include a group I intron type, huge one of 400 or more nucleotides such as M1RNA included in RNaseP, and those called a hammer head type and hair pin type, comprising an active domain as long as 40 nucleotides (Koizumi, M., and Otsuka, E., Protein, Nucleic Acid and Enzyme, 35: 2191, 1990).

For example, a self-cleavage domain of a hammer head type ribozyme digests 3' site of C15 among G13U14C15. The formation of a base pair between U14 and A at 9th is believed to be important for this activity, and the nucleotide at 15th can be digested if it is A or U as well as C (Koizumi, M. et al., FEBS Lett., 228: 225, 1988). If a substrate binding site of a ribozyme is designed to be complementary to an RNA sequence flanking a target site, a restriction enzyme-like ribozyme which recognizes sequences of UC, UU, or UA in a target RNA can be created (Koizumi, M. et al., FEBS Lett., 239: 285, 1988; Koizumi, M., Otuska, E., Protein, Nucleic Acid and Enzyme, 35: 2191, 1990; Koizumi, M. et al., Nucleic Acids Res., 17: 7059, 1989). For example, hundreds of

such sites exist in a coding region of AtNCED3 gene of *Arabidopsis*. A hairpin type ribozyme is found in, for example, a minus strand of a satellite RNA in tobacco ring spot virus (Buzayan, J. M., Nature, 323: 349, 1986). This ribozyme has also been shown to be able to be designed to specifically cleave a target RNA (Kikuchi, Y. and Sasaki, N., Nucleic Acids Res., 19: 6751, 1992; Kikuchi, H., Chemistry and Biology, 30: 112, 1992).

A ribozyme designed for cleaving a target is ligated with a promoter, for example, 35S promoter of cauliflower mosaic viruses and a transcription termination sequence, to be transcribed in a plant cell. When an extra sequence is added at 5' end or 3' end of an transcribed RNA, an activity of a ribozyme may be deleted. In this case, for accurately excising a ribozyme portion alone from a transcribed RNA containing a ribozyme, another trimming ribozyme which works *cis* for trimming at 5' end and 3' end of a ribozyme portion, can be placed (Taira, K. et al., Protein Eng., 3: 733, 1990; Dzianott, A. M. and Bujarski, J. J., Proc. Natl. Acad. Sci. USA., 86: 4823, 1989; Grosshans, C. A. and Cech, R. T., Nucleic Acids Res., 19: 3875, 1991; Taira, K. et al., Nucleic Acids Res., 19: 5125, 1991). In addition, such a constitutive unit is tandemly arranged to cleave multiple sites within a target gene, improving the effect (Yuyama, N. et al., Biochem. Biophys. Res. Commun., 186: 1271, 1992). By using such a ribozyme, a transcript of a target gene of this invention can be cleaved to inhibit its expression. Preferably, a ribozyme specifically cleaves a transcript of a target gene. By using such a ribozyme, a transcript of a target gene of this invention is specifically cleaved to inhibit its expression.

Inhibition of the expression of an endogenous gene can also be achieved by cosuppression due to the introduction of a DNA comprising an identical or a similar sequence to a target gene sequence. "Cosuppression" means a phenomenon in which an introduction of a gene comprising an identical or similar sequence to a target endogenous gene into a plant by transformation inhibits the expression of both an exogenous gene introduced and a target endogenous gene. Details of the mechanism of cosuppression are

not clear, however, it is often observed in plants (Curr. Biol., 7: R793, 1997; Curr. Biol., 6: 810, 1996). For example, a plant in which the expression of a gene encoding a protein having a neoxanthin cleavage activity has been cosuppressed, can be obtained by preparing a vector DNA comprising a gene encoding a protein having a neoxanthin cleavage activity or a similar sequence, transforming an objective plant with the vector, and selecting among obtained plants with a trait in which the expression of a gene encoding a protein having a neoxanthin cleavage activity has been reduced.

A gene to be used for cosuppression does not need to be completely identical to a target gene, however, generally has at least 70% or higher, preferably 80% or higher, more preferably 90% or higher identity. Genetyx (Software Development), a genetic information processing software, can be used for determining an identity or complementarity. This program adopts Lipman-Pearson method (Lipman, D. J. and Pearson, W. R., Science, 227: 1435-1441, 1985). This method first compares sequence data, and calculates identity among the sequences with high homology in consideration with a deletion of a sequence (GAP).

A transformant plant with reduced stress tolerance can be prepared by using a DNA as described above to be used in the present invention that inhibits the expression of a gene. Specifically, the DNA is inserted into an appropriate vector, the vector is introduced into a plant cell, and a transgenic plant is regenerated from the transformant plant cell. As a vector to be used, any vectors can be used in the same manner as in the above case of increasing the stress tolerance as long as an inserted gene can be expressed in a plant cell. Any plant cells can be used to insert a vector. A plant can be a conifer, a broad-leaved tree, a dicot, a monocot, etc. "Plant cell(s)" referred herein include various forms of plant cells, for example, a suspended cultured cell, a protoplast, a leaf slice, a callus, and so on.

Introduction of a vector into a plant cell, and regeneration of a plant from a transformant cell, can be performed by a method known to one skilled in the art, depending on a type of plant cells

in the same manner as in the case of improving stress tolerance. Once a transformant plant in which a DNA of the present invention is introduced into the genome can be obtained, offspring can be obtained from the plant by sexual or asexual propagation. Alternatively, a propagation material (for example, a seed, a fruit, a scicon, a tuber, a tuberous root, a stock, a callus, a protoplast, etc.) can be obtained from the plant, its offspring, or clones, and the plant can be mass-produced from them. The present invention includes a plant cell in which a DNA of present invention is introduced, a plant containing the cell, offspring or a clone of the plant, as well as a propagation material for the plant, its offspring, and clone.

The transformant plant created in such a manner has a reduced ABA content, compared with the wild type plant. Alternatively, the transformant plant has a decreased stress tolerance compared with the wild type plant. The present invention can beapplied to, for example, a weed to effectively eliminate it. In addition, using a inducible promoter as described above, a transformant plant of the present invention can be used for land improvement and such.

The present invention enables creating a plant in which stress tolerance has been increased or decreased. A plant with the improved stress tolerance can grow in harsh land where plants cannot grow thus far. Reduction of stress tolerance can be applied to weeds and such to be eliminated. The method of the present invention can be applied to agriculture to expand cultured area and increase crop yields.

Any patents, patent applications, and publications cited herein are incorporated by reference.

The present invention is illustrated in detail below with reference to examples, but is not to be construed as being limited thereto. Experimental conditions used in the present Examples are as follows.

Growth of cowpea

Seeds of cowpea (*Vigna unguiculata* IT84S-2246-4) were sown

in pots and grown for 8 days in a greenhouse with a photoperiod of 16 hours (in addition to natural light, artificial lighting was supplemented when illumination was insufficient), temperature of 25°C, and appropriate watering.

Dehydration treatment

For dehydration treatment, plants were pulled out of the pot carefully to avoid injury, weighed, and dehydrated on Whatman 3MM filter paper at room temperature and approximately 60% humidity under dim light (300 lux). For the control, plants were pulled out of the pot and immediately transplanted in well-watered soil that was maintained under the same condition for dehydration treatment group.

Analysis of DNA sequence

Plasmid DNA templates were prepared using the Automatic Plasmid Isolation System Model PI-100 (KURABO) and sequenced using the DNA Sequencer Model 373A (ABI). Nucleotide sequences and amino acid sequences were analyzed using a GeneWorks Software System (IntelliGenetics, Inc.), Sequencher 3.0 (Hitachi Software), and the University of Wisconsin Genetic Computer Group (GCG) program.

Example 1: Isolation of cDNA clones corresponding to genes induced by dehydration

A cDNA library was constructed with poly(A)$^+$ RNA that had been isolated from 8-day-old cowpea plants after dehydration stress for 10 hours as follows.

Whole plants were harvested, washed gently to remove soil from the roots and dehydrated on Whatman 3MM filter paper at room temperature and approximately 60% humidity under dim light for 10 hours. Total RNA was prepared from the plants after dehydration treatment by the aforementioned method (Nagy, F. et al., "Analysis of gene expression in transgenic plants." *In* Gelvin and Schilperoort (eds), "Plant Molecular Biology Manual. B4.," Kluwer Academic Publishers, Dordrecht, pp. 1-29, 1988). By

following the reference (Sambrook, J. et al., "Molecular Cloning: A Laboratory Manual," 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), total RNA was passed through an Oligo-dT cellulose column twice to prepare poly(A)$^+$ RNA. About 2% of the RNA applied to the column was collected as the Poly (A)$^+$ RNA fraction. Double-stranded cDNA was synthesized from the Poly (A)$^+$ RNA using cDNA Synthesis System Plus (Amersham Pharmacia Biotech). A cDNA library was constructed from the cDNA using cDNA Cloning System (Amersham Pharmacia Biotech).

The cDNA library was differentially screened with cDNA prepared from poly(A)$^+$ RNA that had been isolated from unstressed cowpea plants and with cDNA prepared from poly(A)$^+$ RNA that had been isolated from plants after dehydration stress for 10 hours. By following the reference (Sambrook, J. et al., "Molecular Cloning: A Laboratory Manual," 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), plaque hybridization was conducted to screen 1 X 10^4 plaques from the cDNA library.

As a result, plaques giving a stronger hybridization signal with [^{32}P]-labeled cDNA were obtained from 10-hour dehydrated cowpea plants. The plasmid regions of the phage clones were excised *in vivo* and used to transform *Escherichia coli* cells. The cDNA fragments from the resultant plasmids were analyzed using the restriction map and the border sequences of the cDNA fragments. From these analyses, cDNA was grouped, and a cDNA clone, named CPRD (CowPea Responsive to Dehydration) 65 could be identified.

Dehydration-induced expression of the gene corresponding to the CPRD65 clone was analyzed by Northern blot hybridization. The 8-day-old plants were pulled out of the soil and dehydrated for various periods up to 12 hours. As controls, similar cowpea plants were pulled out of the soil and were immediately transplanted to well-watered soil. Total RNA was then isolated from dehydrated or control plants for Northern blot hybridization.

Total RNA was isolated according to the method of Nagy et al. (Nagy, F. et al., "Analysis of gene expression in transgenic

plants." *In* Gelvin and Schilperoort (eds), "Plant Molecular Biology Manual, B4.," Kluwer Academic Publishers, Dordrecht, pp, 1-29, 1988), fractionated in a 1% agarose gel containing formaldehyde, and transferred to a nylon filter (Sambrook, J. et al., "Molecular Cloning: A Laboratory Manual," 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). The nylon filter was hybridized with [^{32}P]-labeled CPRD65 cDNA fragments in 50% formamide, 5X SSC, 25 mM sodium phosphate buffer (pH 6.5), 10X Denhardt's solution, and 250 μg/ml of denatured salmon sperm DNA at 42°C. The filter was washed twice with 0.1X SSC, 0.1% SDS at 60°C for 15 min, and subjected to autoradiography.

Figure 1 shows the time course of induction of the expression that corresponds to the CPRD65 gene against dehydration treatment. The expression of CPRD65 was significantly increased by dehydration stress. The mRNA corresponding to the CPRD65 was observed to accumulate within 2 hours after the initiation of dehydration treatment.

Cowpea plants dehydrated for 10 hours appeared wilted. These wilted plants showed recovery from wilting within 4 hours after transfer to well-watered soil (rehydration treatment). After rehydration, the level of CPRD65 mRNA decreased (Fig. 1). The CPRD65 gene exhibited typical and significant responses to drought stress, namely, induction of the transcriptions by dehydration and reduction of the level upon rehydration. These facts suggested that the CPRD65 gene is involved in drought tolerance.

Example 2: Sequence analysis of the CPRD65 cDNA

Since the CPRD65 cDNA fragment isolated in Example 1 was not possibly full length, the same cDNA library was screened again with a partial CPRD65 cDNA as a probe to isolate a full-length cDNA (SEQ ID NO: 11). An amino acid sequence encoded by the full-length cDNA clone (SEQ ID NO: 12) was shown in Fig. 2. The full-length CPRD65 cDNA consists of 2432 bp, including a 5'-flanking region of 125 bp and 3'- flanking region of 486 bp. One polyadenylation consensus sequence (AATAAA) was found in the

3'-flanking region. This sequence has an open reading frame encoding a polypeptide of 612 amino acids with a calculated molecular weight for the putative protein of 67.6 kDa. Comparison of the deduced amino acid sequence of the CPRD65 protein with the protein database revealed an extensive homology with VP14 from maize (*Zea mays*) (61%) (Schwartz, S. H., Science, 276: 1872-1874, 1997) and a neoxanthin cleavage enzyme from tomato (*Lycopersicon esculentum)* (69%), (Burbidge, A. et al., J. Exp. Bot., 47: 2111-2112, 1997; Burbidge, A. et al., Plant J., 17: 427-431, 1999) as shown in Fig. 2. The putative CPRD65 protein seems to contain a transit polypeptide in its N-terminal region like the VP14 protein. The N-terminal sequences of the CPRD65, VP14, and tomato neoxanthin cleavage enzyme have low sequence similarity, but structural similarity.

Example 3: Genomic Southern blot analysis of the CPRD65 gene

In order to analyze genes related to the CPRD65 of cowpea plants, genomic Southern blot hybridization was conduced in the conditions of two stringencies (Fig. 3).

The genomic Southern blot analysis was conducted according to the method of the reference (Sambrook, J. et al., "Molecular Cloning: A Laboratory Manual," 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). Genomic DNA of 10 μg was digested with restriction enzymes, separated in a 1% agarose gel, and blotted to a nylon filter. The filter was hybridized with [^{32}P]-labeled fragments in 30% formamide, 6X SSC, 5X Denhardt's solution, and 100 μg/ml of denatured salmon sperm DNA at 42°C. The filter was washed twice with 0.1X SSC, 0.1% SDS at 60°C for 15 min (B), or washed twice with 0.5X SSC, 0.5% SDS at 37°C for 15 min (A), and subjected to autoradiography.

The CPRD65 cDNA had no internal restriction site for EcoRI and XbaI and had two flanking internal restriction sites for HindIII, confirmed by its nucleotide sequence. One hybridized band in the EcoRI and XbaI digest and two hybridized bands in the Hind III digest were detected using the CPRD65 cDNA as a probe. Some additional faint hybridized bands were detected under the

above stringency condition (A). These results suggest that the CPRD65 gene constitutes a small gene family with related genes.

Example 4: Northern blot analysis of the CPRD65 gene

The effects of various environmental stresses on the expression of the CPRD65 gene were analyzed. For high salinity, ABA, and water treatments, plants were pulled out of the soil in the same manner as in the dehydration treatment, and grown by the hydroculture in the solutions containing 250 mM NaCl, 100 μM ABA, and deionized water, respectively. For heat and cold treatments, potted plants were transferred to the incubators at 40°C and 4°C, respectively. Each stress treatment to plants was conducted for 0, 1, 2, 5, 10, and 24 hours. After the treatments, the treated plants were immediately frozen with liquid nitrogen, and the RNAs were isolated for Northern blot analysis.

As a result, it was found that the expression of this gene was strongly induced under a high-salt condition, but not by cold or heat stress (Fig. 4A). The induction of the CPRD65 gene was not detected by ABA treatment or water treatment.

To determine the tissue specificity of the expression of the CPRD65 gene under drought stress, Northern blot hybridization of total RNA prepared from leaves, stems, or roots under a normal or drought condition was performed (Fig. 4B). The CPRD65 transcript was strongly induced in stems and leaves by drought treatment, but less in roots.

Example 5: Enzymatic activity of the bacterially expressed CPRD65 protein

The deduced amino acid sequence of the CPRD65 gene has high homology with an amino acid sequence of a neoxanthin cleavage enzyme encoded by the maize VP14 gene (Fig. 2). To examine whether the CPRD65 gene encodes a neoxanthin cleavage enzyme, the biochemical properties of the recombinant CPRD65 protein expressed in *E. coli* were analyzed. A DNA fragment for the CPRD65 coding region was amplified by PCR and fused to the GST gene in frame using the pGEX4T-1 (Pharmacia) to construct a chimeric

plasmid pGST-CPRD65 as follows.

The DNA encoding the CPRD65 protein was amplified by PCR using primers: 5'-ATTGAATTCATGCCTTCAGCTTCAAAC-3' (SEQ ID NO: 19) and 5'-ATTGGATCCCAAAAGCTACACGCTGGTCCCC-3' (SEQ ID NO: 20). The PCR fragment was inserted into the EcoRV site of pBluescript II SK$^+$ vector. Sequences of the inserted PCR fragments were confirmed to determine whether a mutation was generated in a nucleotide sequence by PCR. The PCR fragment in which any mutation was not identified in the nucleotide sequence was isolated from the pBluescript II SK$^+$ vector as a DNA fragment using restriction enzymes (EcoRI and XhoI) and inserted into the EcoRI to XhoI site of pGEX4T-1 (Amersham Pharmacia Biotech) to construct pGST-CPRD65. Cells of *Escherichia coli* strain JM109 were transformed with pGST-CPRD65 or pGEX4T-1 and cultured in L broth at 37°C. When OD_{600} reached about 0.5, isopropyl β -D-thiogalactopyranoside (IPTG) was added, and incubation was continued for 12 hours at 17°C. The cells were harvested, washed, and suspended in extraction buffer [10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5% glycerol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM dithiothreitol (DTT)]. The procedures for purification of the fused protein and digestion with thrombin were performed according to the instruction manual for the GST gene fusion system (Amersham Pharmacia Biotech). The protein concentration was determined with a protein assay kit (Bio-Rad).

The GST-CPRD65 fusion protein was overexpressed in *E. coli* in the manner described above, and purified from the crude cell extract using a glutathione-Sepharose 4B. Whether this purified GST-CPRD65 recombinant protein digests *cis*-neoxanthin, *trans*-violaxanthin, and *cis*-violaxanthin to produce xanthoxin was examined.

The assay procedures for neoxanthin cleavage enzyme activity have been described (Schwartz, S. H. et al., Science, 276: 1872-1874, 1997). *cis*-Neoxanthin and *trans*-violaxanthin were prepared from spinach leaves. *cis*-Violaxanthin was prepared from orange peel. The reaction mixture (100 μl) contained 100 mM Bis-Tris (pH 6.7), 0.05% Triton X-100, 10 mM ascorbic acid,

5 mM $FeSO_4$, and a protein sample. The reaction was allowed to proceed at room temperature for 1 hour. After addition of 1 ml of water, the reaction mixtures were extracted with *n*-hexane (1 ml x 2) and then ethyl acetate (1 ml x 2). The *n*-hexane fraction was concentrated and submitted to HPLC analysis on a column of Nucleosil 5 C_{18} (150 mm length, 8 mm internal diameter (i. d.)). The column was eluted with a linear gradient between solvent A (85% ethanol) and solvent B (chloroform and methanol, 1:1) at a flow rate of 1.5 ml/min. The concentration of solvent B was increased from 10% to 50% in 25 min, and kept at 50% for 5 min. The absorbance of the eluate was monitored with a UV detector at 440 nm. The ethyl acetate fraction was purified with HPLC on a column of Nucleosil 5 C_{18} (150 mm length, 8 mm i. d.). The column was eluted with 50% aqueous methanol at a flow rate of 1.5 ml/min, and the absorbance of the eluate was monitored with a UV detector at 260 nm. The predicted xanthoxin fraction was collected and submitted to GC-MS analysis. In each step, samples were shielded from light as much as possible.

GC-MS analysis was conducted as follows. An AUTOMASS mass-spectrometer (Nippon Denshi) equipped with a 5890 gas chromatography (Hewlett Packard) was used for the analysis. The analytical conditions were as follows: ionization, EI 70 eV; column, DB-5 (15 m length; 0.25 mm i. d.; 0.25 μm film thickness; J&W Scientific); carrier gas, He (1 ml min^{-1}); injection temperature, 250°C; transfer line temperature, 250°C; and initial heating temperature, 80°C. Starting 1 min after injection, the heating temperature was increased to 200°C at a rate of 30°C min^{-1} followed by further increment to 230°C at a rate of 5°C min^{-1}.

As shown in Fig. 5, the predicted C25-product and xanthoxin were detected in the reaction mixture with the GST-CPRD65 protein and *cis*-neoxanthin by HPLC analysis. The occurrence of xanthoxin was confirmed by GC-MS analysis in which ions characteristic to xanthoxin were observed. The ions and their relative intensities were: m/z 250 (4), 168 (32), 149 (77), 107 (61), and 95 (100). Xanthoxin and C25-product were not formed from *trans*-violaxanthin (data not shown). These results were not affected by the

treatment with thrombin which separates the GST-CPRD65 recombinant protein into GST and CPRD65 portions.

Example 6: Analysis of N terminal region of the CPRD65 protein as a transit peptide in protoplasts prepared from *Arabidopsis*

The N-terminal region of the CPRD65 protein has typical structural features of transit peptides that are involved in chloroplast targeting. This structural feature of the CPRD65 protein suggests that the mature CPRD65 protein is located in plastids including chloroplasts. To analyze the role of its N-terminal region as a transit peptide, a chimeric gene 35S::CPRD65N-sGFP that encodes the N-terminal region of the CPRD65 protein (1-148) between the CaMV 35S promoter and the synthetic green florescent protein (sGFP) gene of the jellyfish *Aequorea victoria* (Chiu, W., et al., Curr. Biol., 6: 325-330, 1996) was constructed.

The DNA corresponding to the N-terminal peptide (1 to 148 amino acids) of the CPRD65 protein was amplified by PCR using primers: 5'-ATATATCTAGAATGCCTTCATCAGCTTCAAACACTTGG-3' (SEQ ID NO: 21) and 5'-ATATAGGATCCCTCCGGCACCGGCGCGAAGTTCCCG-3 (SEQ ID NO: 22). The PCR fragment was inserted into the pBluescript II SK$^+$ vector and verified to have no sequence mutation caused by PCR. The DNA fragment was inserted into the site between 35S-promoter and sGFP gene on transient expression vector (Chiu, W. et al., Curr. Biol., 6: 325-330, 1996). The preparation, DNA transfection, and incubation of the *Arabidopsis* protoplasts were performed as previously described (Abel, S. and Theologis, A., Plant J., 5: 421-427, 1994).

35S::CPRD65N-sGFP fusion construct and its control construct (35S::sGFP) were introduced into protoplasts prepared from *Arabidopsis* by a DNA-transfection method (Abel, S. and Theologis, A., Plant J., 5: 421-427, 1994). The protoplasts were observed by fluorescent microscopy 2 to 4 days after the transformation. As shown in Fig. 6, when 35S::CPRD65N-sGFP was transiently expressed in the protoplasts, fluorescence was localized in plastids. On the other hand, when 35S::sGFP

construct was introduced, fluorescence was detected not in plastids, but mainly in the cytoplasm. These results suggest that N-terminal region of the CPRD65 protein functions as a transit peptide to target the CPRD65 protein into the plastids. The CPRD65 protein was expected to be localized in plastids, and function to produce ABA in plastids.

Example 7: Accumulation of ABA by dehydration stress in 8-day-old cowpea plants.

The accumulation of endogenous ABA level in a 8-day-old cowpea plant was measured by dehydration conditions.

Samples were homogenized in liquid nitrogen and extracted with aqueous methanol (20 to 80 %) twice. After addition of [$^2H_3$]ABA, the extracts were concentrated, and submitted to a standard solvent fractionation procedure to give an acidic-ethyl acetate soluble fraction. It was purified using Bond Elut cartridge (C_{18} and DEA, Varian) by the procedure reported previously (Wijayanti, L., et al., Biosci. Biotech. Biochem., 59: 1533-1535, 1995). Purified samples from undesiccated plants were then subjected to HPLC analysis with a Senshu Pak ODS-2101-N column (100 mm length, 6 mm i. d., Senshu Scientific Co.). The analytical conditions were the same as reported previously (Wijayanti, L., et al., Biosci. Biotech. Biochem., 59: 1533-1535, 1995). Samples thus purified were methylated with etherial diazomethane and submitted to GC-SIM analysis.

As shown in Fig. 7, ABA began to accumulate within 2 hours after dehydration. The level of ABA in 10-hour dehydrated plants was 140 times higher than that in unstressed control plants. The timing of accumulation of the CPRD65 mRNA was earlier than that of ABA mRNA accumulation (Fig. 7).

The expression of the CPRD65 gene was strongly induced by drought stress in leaves and stems, but slightly in roots (Fig. 4B). The relationship between the expression of the CPRD65 gene and the accumulation of endogenous ABA under drought stress was examined. The 8-day-old cowpea plants were separated into leaves, stems, and roots, and then dehydrated. The endogenous ABA levels

in these organs were measured before or after dehydration treatment. As shown in Fig. 8, endogenous ABA were dramatically accumulated by drought stress in leaves and stems, but slightly in roots. The tissue-specific pattern of ABA accumulation under drought stress was consistent with that of the expression of the CPRD65 gene as shown in Figs. 4B and 8.

Example 8: Analysis of xanthophylls in cowpea leaf

Xanthophylls in cowpea leaf were analyzed to find possible substances for the CPRD65 protein.

Samples were extracted with acetone twice, and the extracts were concentrated, dissolved in 80% methanol (1 ml), and loaded onto a Bond Elut C_{18} column. The column was washed with additional 4 ml of 80% methanol, and xanthophylls were eluted with 5 ml of methanol-water-chloroform (71:9:20). The eluate was concentrated and applied to HPLC analyses with columns of Nucleosil 5 C_{18} and Senshu Pak Silica-2251-S (250 mm length, 6 mm i. d.). Conditions for ODS-HPLC were the same as described above. For Silica-HPLC, a flow rate of 1.5 ml/min and a linear gradient of solvent B concentration from 10% to 100% in 30 min were used where solvent A was ethyl acetate-n-hexane (1:1) and solvent B is ethyl acetate. The xanthophylls were identified from their visible and ultraviolet spectroscopic data.

trans-Neoxanthin, *trans*-violaxanthin, and *cis*-neoxanthin were detected as major xanthophylls, and *cis*-violaxanthin was detected as a minor component in cowpea leaf by optical spectroscopic analysis of visible and ultraviolet lights (data not shown). The endogenous amounts of *trans*-neoxanthin, *trans*-violaxanthin, and *cis*-neoxanthin were not significantly different between under normal growth conditions and drought conditions.

As shown above, cowpea drought-inducible CPRD65 gene encodes the neoxanthin cleavage enzyme, and its product is localized in plastids. The CPRD65 gene was strongly induced mainly in leaves and stems under drought and high salt conditions. Strong accumulation of ABA in leaves and stems under drought

conditions was observed, which showed a similar pattern of the CPRD65 gene expression. These results strongly suggest that the CPRD65 protein is an enzyme mainly involved in the ABA biosynthesis under drought stress in cowpea plants.

Example 9: Isolation of *Arabidopsis* cDNA clone encoding a homolog of neoxanthin cleavage enzyme gene

Using the CPRD65 as a probe, a cDNA library of *Arabidopsis* (Abe H. et al, Plant Cell, 9: 1859-1868, 1997) was screened. As a result, many plaques indicating strong hybridization signals were obtained. From these plaques, phage clones were isolated, and a cDNA region was excised with restriction enzymes to insert it into a pBluescript SK+ and transfect *E. coli*. By analysis of a DNA sequence, these clones were classified into one group. This was designated AtNCED3. The AtNCED3 showed a significant homology with the CPRD65 encoding a neoxanthin cleavage enzyme (Fig. 9). A nucleotide sequence of the AtNCED3 cDNA and an amino acid sequence of the AtNCED3 protein are shown in SEQ ID NOs: 5 and 6, respectively.

Example 10: Isolation of the AtNCED1, 2, 4, and 5, and analysis of phylogenic tree

Search on DNA databases using the nucleotide sequences of the CPRD65 and the AtNCED3 identified four sequences with high homology (Ac. No. AL021713, AL021687, AJ005813, AB028621).

In order to isolate a gene with high homology existing in the AL021713 sequence, a target gene fragment was amplified by the PCR method using a gDNA as a template, and 5'-CCCGGGATCCCTCAAGCCTCTCTATACCG-3' (SEQ ID NO: 23) and 5'-CCCGGGATCCTTTATACGGATTCTGAGGGAG-3' (SEQ ID NO: 24) as primers. Using the fragment as a probe, a clone containing the target gene was isolated from the gDNA library (Clontech). The gene was amplified again by the PCR method using 5'-CCCGGGATCCCTCAAGCCTCTCTATACCG-3' (SEQ ID NO: 23) and 5'-CCCGGGATCCTTTATACGGATTCTGAGGGAG-3' (SEQ ID NO: 24) as primers, and cloned into the EcoRV site of pBluescript II SK$^+$ (Stratagene)

to determine a nucleotide sequence. This gene was designated AtNCED1. A nucleotide sequence of the AtNCED1 cDNA and an amino acid sequence of the AtNCED1 protein are shown in SEQ ID NOs: 1 and 2, respectively.

In order to isolate a gene with high homology existing in the AL021687 sequence, a target gene fragment was amplified by the PCR method using a gDNA as a template, and 5'-ATTGAATTCATGGACTCTGTTTCTTCTTCTTCC-3' (SEQ ID NO: 25) and 5'-ATTGAATTCTTAAAGCTTATTAAGGTCACTTTCC-3' (SEQ ID NO: 26) as primers. Using the fragment as a probe, a clone containing the target gene was isolated from the gDNA library (Clontech). The gene was amplified again by the PCR method using 5'-ATTGAATTCATGGACTCTGTTTCTTCTTCTTCC-3' (SEQ ID NO: 25) and 5'-ATTGAATTCTTAAAGCTTATTAAGGTCACTTTCC-3' (SEQ ID NO: 26) as primers, and cloned into the EcoRV site of pBluescript II SK+ (Stratagene) to determine a nucleotide sequence. This gene was designated AtNCED2. A nucleotide sequence of the AtNCED2 cDNA and an amino acid sequence of the AtNCED2 protein are shown in SEQ ID NOs: 3 and 4, respectively.

In order to isolate a gene with high homology existing in the AJ005813 sequence, a target gene fragment was amplified by the PCR method using a gDNA as a template, and 5'-AAGAATTCATGGCGGAGAAACTCAGTGATGGCAGC-3' (SEQ ID NO: 27) and 5'-AAAAGAATTCGGCTTATATAAGAGTTTGTTCCTGG-3' (SEQ ID NO: 28) as primers. Using the fragment as a probe, a clone containing the target gene was isolated from the cDNA library (Clontech). The gene was amplified again by the PCR method using 5'-AAGAATTCATGGCGGAGAAACTCAGTGATGGCAGC-3' (SEQ ID NO: 27) and 5'-AAAAGAATTCGGCTTATATAAGAGTTTGTTCCTGG-3' (SEQ ID NO: 28) as primers, and cloned into the EcoRV site of pBluescript II SK+ (Stratagene) to determine a nucleotide sequence. This gene was designated AtNCED4. A nucleotide sequence of the AtNCED4 cDNA and an amino acid sequence of the AtNCED4 protein are shown in SEQ ID NOs: 7 and 8, respectively.

In order to isolate a gene with high homology existing in the AB028621 sequence, DNA was isolated from P1 clone MUJ8. The

gene was amplified by the PCR method using 5'-CGGGATCCATGCAACACTCTCTTCGTTCTGATCTTCTTC-3' (SEQ ID NO: 29) and 5'-CGGGATCCTCAGAAAACTTGTTCCTTCAACTGATTCTCGC-3' (SEQ ID NO: 30) as primers, and cloned into the EcoRV site of pBluescript II SK+ (Stratagene) to determine a nucleotide sequence. This gene was designated AtNCED5. A nucleotide sequence of the AtNCED5 cDNA and an amino acid sequence of the AtNCED5 protein are shown in SEQ ID NOs: 9 and 10, respectively.

Figure 10 shows the alignments of the amino acid sequences of the AtNCED1 to 5. To examine relationship between amino acid sequences deduced from each sequence and sequences on the databases, the phylogenic tree analysis was conducted (Fig. 11). A phylogenic tree was constructed using GeneWorks (Intelligenetics, Inc.), a software for analyzing genes. Algorithm used UPGMA (Unweighted Pair Group Method with Arithmetic Mean: Molecular Evolutionary genetics, written by Nei, M., translated by Gojo, H. and Saito N., Baifukan, pp. 252-256, Japan).

Example 11: Northern blot analysis of the AtNCED genes

Effects of various environmental stresses on the expression of each identified AtNCED gene were analyzed by Northern blot analysis.

Plants grown on an agar plate for three weeks were used for each stress treatment. For dehydration stress, plants were pulled out of an agar medium, and air-dried on filter paper (relative humidity 50%). For salt stress, ABA treatment, and water treatment as a control, plants were pulled out and placed in a Petri dish containing 250 mM NaCl solution, 100 μM ABA solution, and distilled water, respectively, so that only roots were immersed, for a certain period of time at room temperature with a lid closed. For cold and heat stresses, agar plates were placed in a constant-temperature incubator at 4°C and 40°C, respectively, for a certain period of time.

The plants treated with each environmental stress above were crushed in liquid nitrogen, total RNA was extracted (Nagy

F, Kay SA and Chua N-H (1988) Analysis of gene expression in transgenic plants. *In* Gelvin and Schilperoort (eds), Plant Molecular Biology Manual, B4. Kluwer Academic Publishers, Dordrecht, pp 1-29), and 20 μg each of samples were loaded on each lane of a 1% agarose gel and electrophoresed. The RNA was blotted from the gel to a nylon membrane after the electrophoresis, and subjected to Northern hybridization using a [^{32}P]-labeled cDNA probe (Sambrook, J., Fritsch EF and Maniatis T (1989) Molecular Cloning: A Laboratory manual, 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.).

As a result, it was found the AtNCED3 gene expression was strongly induced by drought, high salt concentration, and cold condition. Heat condition did not induce the expression. In addition, for ABA treatment or water treatment, the induction of the AtNCED3 gene expression was not detected (Fig. 12).

Example 12: Enzymatic properties of the bacterially expressed AtNCED protein

The deduced amino acid sequence of the AtNCED3 gene has high homology with that of the cowpea CPRD65 gene encoding a neoxanthin cleavage enzyme (Fig. 9). To examine whether the AtNCED3 gene encodes a neoxanthin cleavage enzyme, the biochemical properties of the recombinant AtNCED3 protein expressed in *E. coli* were analyzed.

The DNA encoding the AtNCED3 protein was amplified by PCR using the cloned AtNCED3 cDNA as a template, and 5'-ATTGAATTCATGGCTTCTTTCACGGCAACGGC-3' (SEQ ID NO: 31 and 5'-GTTTTCCCAGTCACGAC-3' (SEQ ID NO: 32) as primers. The PCR fragment was cloned into the EcoRV site of pBluescript II SK$^+$ (Stratagene). Sequences of the inserted PCR fragments were confirmed to determine whether a mutation was generated in a nucleotide sequence by PCR. The DNA fragment in which any mutation was not identified was cloned in frame into the EcoRI site of pGEX4T-1 containing glutathione S-transferase (GST) gene (Amersham Pharmacia Biotech) to construct chimeric plasmid pGST-AtNCED3. Cells of *Escherichia coli* strain JM109 were transformed with

pGST-AtNCED3 or pGEX4T-1 and cultured in L broth at 37°C. When CD_{600} reached about 0.5, isopropyl β-D-thiogalactopyranoside (IPTG) was added, and incubation was continued for 12 hours at 17°C. The *E. coli* cells were harvested, washed, and suspended in extraction buffer [10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5% glycerol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM dithiothreitol (DTT)]. The procedures for purification of the fused protein and digestion with thrombin were performed using glutathione-Sepharose 4B [the GST gene fusion system (Amersham Pharmacia Biotech)] according to its instruction manual. The protein concentration was determined with a protein assay kit (Bio-Rad, CA, USA).

As a result of assays for the neoxanthin cleavage enzyme activity, expected C25 product and xanthoxin were detected in the reaction mixture containing GST-AtNCED3 protein and *cis*-neoxanthin, confirming that the AtNCED3 protein comprises a neoxanthin cleavage activity. The similar experiment detected a neoxanthin cleavage activity in the AtNCED1 and AtNCED5.

Example 13: Preparation of transgenic plants

Arabidopsis (*Arabidopsis thaliana* (L.) Heynh. ecotype Columbia) was used as a sample. Wild type *Arabidopsis* plants were sown in a 9 cm-diameter plastic-pot with culture soil, grown for 6 weeks at 22°C with a photoperiod of 16-hours, and then used for transformation.

A vector without a GUS reporter gene (pBE2113NOT) was constructed from a pBE2113 vector with a kanamycin resistant marker and a 35S promoter of cauliflower mosaic virus (Mitsuhara, I. et al., Plant Cell Physiol., 37: 49-59, 1996), and the cDNA of the AtNCED3 isolated from *Arabidopsis* was ligated to the vector at BamHI site in the right direction (a sense direction) or the opposite (an antisense direction). The obtained vectors were introduced into a soil bacterium (*Agrobacterium tumefaciens* strain GV3101 (pMP90)) by mixing the vectors with the bacterium. The *Agrobacterium tumefaciens* with the target gene was selected by kanamycin (Km) resistance, and infected wild type *Arabidopsis*

plants using the vacuum infiltration method (Bechtold, N. et al., C. R. Acad. Sci. Paris, Life Sci., 316: 1194-1199, 1993). From the infected plants, dry seeds were harvested, sown on an agar plate supplemented with Km, and grown to select individuals of the transformant first generation (T1). Seeds of transformant second generation (T2) obtained from the transformant first generation were sown on an agar plate supplemented with Km, and grown to collect seeds for the third generation (T3) from plants showing Km resistance. Moreover, seeds of the third generation were sown on a plate supplemented with Km in the similar manner, and those of all seeds showing drug resistance were used for the following experiments as a T3 homologous line. Finally, two lines for each sense and antisense transformant of the AtNCED3 gene were isolated.

Example 14: Evaluation of the expression of the AtNCED3 gene in transformants

The expression of the AtNCED3 gene in wild type *Arabidopsis* and AtNCED3 gene-transformant plants was evaluated by Northern hybridization method.

Plants cultivated for a month were used for analyses of the AtNCED3 gene expression in transformants. The plants were pulled out and air-dried on filter papers as drought stress (relative humidity 50%). Plants that bore the environmental stress treatment above were broken in liquid nitrogen to extract total RNA (Nagy, F., Kay, S. A. and Chua, N. -H. (1988) Analysis of gene expression in transgenic plants. In Gelvin and Schilperoort, eds, Plant Molecular Biology Manual, B4. Kluwer Academic Publishers, Dordrecht, pp 1-29). The RNA was electrophoresed (20 μg per lane) on 1% agarose gel, transferred from the electrophoresed gel onto a nylon membrane, and subjected to Northern hybridization by using a [^{32}P]-labeled RNA probe (Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.).

As a result, the AtNCED3 gene was not expressed in the wild type before the drought stress but already expressed in the sense

plants. On the other hand, after the drought treatment, the AtNCED3 gene was induced to express by drought in the wild type plants but not expressed in the antisense plants even after the drought stress treatment (Fig. 13).

Example 15: Evaluation of endogenous ABA amount in transformants

Endogenous ABA amounts were measured in wild type *Arabidopsis* and AtNCED3 gene-transformant plants.

Plants cultivated for a month were used for evaluation of endogenous ABA amounts in wild type *Arabidopsis* and transformants. Samples were homogenized in liquid nitrogen and extraction with aqueous methanol (20 to 80%) was performed twice. After adding [$^2H_3$] ABA, extracts were concentrated, and acidic-ethyl acetate soluble fractions were obtained by a standard solvent fractionation. These fractions were purified using Bond Elut cartridge (C_{18} and DEA, Varian) by following the method described in Wijayanti, L. et al., Biosci. Biotech. Biochem., 59: 1533-1535, 1995. Samples purified from undehydrated plants were analyzed using Senshu Pak ODS-2101-N column (100 mm length, 6 mm i. d.) (Senshu Scientific Co.) by HPLC. Analytic conditions were the same as described in Wijayanti, L. et al., Biosci. Biotech. Biochem., 59: 1533-1535, 1995. Purified samples were methylated by etherial diazomethane and analyzed by GC-SIM.

As a result, the ABA amount increased in the sense plant compared with its wild type and decreased in the antisense plant (Fig. 14).

Example 16: Results of evaluation of drought tolerance

The seeds of the obtained transformant plants were sown on an agar plate supplemented with nutritive salts (Valvekens, D. et al., Proc. Natl. Acad. Sci. USA, 85: 5536-5540, 1988), and grown under the above growing condition for two weeks to subject to the following experiment.

Four individuals of the above plants were transplanted to plastic pots with a diameter of 9 cm filled with the soil (vermiculite:perlite = 1:1) and grown under the condition with

temperature of 22°C and a photoperiod of 16 hours. Three weeks after sowing seeds (two weeks after the transplantation), the pots with the plants were dehydrated by stopping watering to naturally give drought stress. Fourteen days and 17 days after the initiation of non-irrigation, the pictures of the plants were taken. The plants in which the AtNCED3 gene was introduced in the antisense direction wilted 14 days after the initiation of non-irrigation (Fig. 15). In contrast, the transformant plants in which the gene was introduced in the sense direction seldom wilted. The wild lines also wilted 17 days after the initiation of non-irrigation, while the transformant plants in which the gene was introduced in the sense direction showed significant tolerance to drought (Fig. 16).